Antiproliferative and Differentiative Effect of Granulocyte-Macrophage Colony-stimulating Factor on a Variant Human Small Cell Lung Cancer Cell Line

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ABSTRACT

A variant clone was adopted during passages of a small cell lung cancer cell line, GKT3-1.3. The variant clone exhibited distinct characteristics with alterations in morphology, positive staining with nonspecific esterase stain, and an increase in specific surface markers OKM5, HLA-DR, Mo1, and My7, usually found on monocytes or their precursors. However, it exerted a very rapid proliferation just like immature cells. This new clone, GKT3-1.3V, was shown to have specific binding capacity to granulocyte-macrophage colony-stimulating factor (GM-CSF), with a number of binding sites comparable to that of myelomonocytes or monocyctic cell lines. Thus its proliferation was inhibited by GM-CSF in clonogenic assay and suspension culture. Increase in the percentage of cells with surface marker Mo1 by the addition of GM-CSF suggested its differentiative effect. Cell cycle analysis showed that the antiproliferative effect of GM-CSF was due to a block in G0 or G1. The antiproliferative effect of GM-CSF was abolished by the addition of anti-GM-CSF antibody.

INTRODUCTION

SCLC has distinct clinicopathological characteristics with high rates of metastases and poor prognosis in spite of its high sensitivity to chemotherapy or radiation. There has been a debate on the cellular origin of SCLC. It has been hypothesized that SCLC derives from pulmonary epithelial cells that differentiate along a neuroendocrine pathway in the fetal bronchi (1). Alternatively there has been another hypothesis that it arises from macrophages of bone marrow origin (2). Continuous SCLC cell lines (3-6) and their variants with altered morphological features, growth pattern, and biochemical properties (7, 8) have been successfully established. These established cell lines have enabled us to study the biological characteristics of SCLC. The presence of a series of monocyte-specific surface antigens have been shown on some of these cell lines. This finding supported the latter hypothesis that their origin is related to cells of myeloid derivation, such as macrophages or their precursors (9-12). In addition, γ-interferon and GM-CSF have been reported to induce the expression of myeloid-associated surface antigens and inhibit the proliferation of the SCLC cells (13).

In the present study, we further tested the effects of other hematopoietic growth factors, M-CSF, and G-CSF, in addition to GM-CSF, on a SCLC cell line, GKT3-1.3, and its variant, GKT3-1.3V, which exhibited monocyte-like properties. Among these CSFs only GM-CSF exhibited an antiproliferative effect in clonogenic assay and suspension culture assay. The antiproliferative effect was associated with a block in the cell cycle, together with a differentiative effect on the surface marker expression. These effects were considered to be the direct activity of GM-CSF bound to the cells.

MATERIALS AND METHODS

Cell Lines. The cell line GKT3 was kindly provided by Dr. Shibuya, Tokyo University Research Institute of Medical Sciences. The cell line was established from the specimen obtained by autopsy from the metastatic lesion in liver of a SCLC patient (4). Three subclones, GKT3-1.1, -1.2, and -1.3, were propagated at first in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% FCS (GIBCO) and later in RPMI 1640 (GIBCO), with 10% FCS. The cells proliferated in suspension as floating aggregates. During approximately 5 months of continuous culture, one subclone GKT3-1.3 gradually changed its characteristics with a culture pattern of more loosely associated clusters and a much more rapid doubling time. Morphological changes were also observed by microscopic examinations: increased amount of cytoplasm and appearance of distinct large nuclei. This variant clone was designated as GKT3-1.3V. The cells were positive for α-naphthyl butyrate esterase stain which was inhibited by NaF, while the parent cells GKT3-1.3 were negative for the stain.

Biological Reagents. CSFs used in the present study were: rGM-CSF (Sumitomo Pharmaceutical Co., Takarazuka, Japan) (14), rM-CSF (Otsuka Pharmaceutical Co., Tokushima, Japan); the rM-CSF was obtained as a medium conditioned by monkey COS cells transfected with the expression vector containing complementary DNA for M-CSF cloned from human T-cell line CEM-ON. M-CSF was expressed as units and 1 M-CSF unit is defined as the activity that produces a single colony consisting of more than 50 cells from 1.5 × 10⁵ murine bone marrow cells and rG-CSF (Chugai Pharmaceutical Co., Toshiba-ku, Tokyo, Japan) (15). Anti-human GM-CSF antibody was obtained from the Genetics Institute (Cambridge, MA), which had been raised in sheep with recombinant Chinese hamster ovary cell-derived GM-CSF.

Surface Marker Analysis. Cell surface antigens were analyzed by a FACScan (Becton Dickinson, Sunnyvale, CA), equipped with logarithmic amplifiers and a 488-nm argon ion laser, as described previously (16). The monoclonal antibodies used were: OKM5 (CD36), HLA-DR, Mo1 (CD11), My4 (CD14), My7 (CD13), Leu-7. Colony Assay. Cells were plated at 5-10 × 10⁴ cells/ml in RPMI 1640 with 0.8% methylcellulose (4000 cps; Wako Co., Osaka, Japan) and 15% FCS as described previously (17, 18). The effects of rGM-CSF, rM-CSF, and rG-CSF on colony growth were assayed by continuous addition of these agents in this system for 6 to 7 days of incubation at 37°C in 5% CO₂. Colonies (more than 40 cells) were enumerated under an inverted microscope.

Suspension Culture. Cells (1-2 × 10⁴) were cultured in 1 ml of RPMI 1640 with 15% FCS with or without the addition of CSFs for different periods of time and then harvested, counted, washed three times, and used for either subculture or colony assay. The recovery of clonogenic cells were determined by multiplying the number of cells harvested per dish from suspension by the plating efficiency in methylcellulose (19).

Cell Cycle Analysis. Cell cycle analysis was performed as described previously (20). The content of DNA was assayed by flow cytometry with a FACS-440 with collection of fluorescence emission longer than 590 nm. More than 10⁵ cells were counted and the distribution histo-
grams of fluorescence intensity in linear scale were obtained. Cell cycle analysis by DNA distribution was performed using the CCANA program reported by Dean (21), and the populations in G1, S, and G2-M phase were calculated.

Receptor Assay. GKT3-1.3V cells were washed with cold binding buffer (RPMI 1640 supplemented with 0.1% bovine serum albumin (Sigma, St. Louis, MO) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma)); Cells, 3.75 × 10^6 in 200 μl of binding buffer containing 125I-GM-CSF (specific activity, 1.28 × 10^6 cpm/ng), at various concentrations with or without a 50-fold excess of unlabeled GM-CSF were incubated for 1.5 h at 20°C. At the end of the incubation, duplicate 80-μl aliquots removed from the incubation mixture were layered on 100 μl of di-n-butyl phthalate (Wako Pure Chemical Industries, Osaka, Japan) and centrifuged for 2 min at 10,000 × g to separate bound and free 125I-GM-CSF. The cell pellets were counted for radioactivity by a gamma counter. Radioactivity bound to the cells in the presence of 50-fold excess of unlabeled GM-CSF was considered as nonspecific binding and was subtracted from the total binding (22, 23).

RESULTS

Surface Markers. Table 1 indicates the differences of surface markers between the CKT3-1.3 and GKT3-1.3V cells, together with the influences on the surface markers of the continuous addition of CSFs for 3 days of incubation. OKM5, HLA-DR, Mo1, and My7, which are markers present on monocytes, were proved positive for the variant cells. These findings, in addition to the morphological appearance and staining for nonspecific esterase (see "Materials and Methods"), indicate the possibility of mutation or differentiation of GKT3-1.3 cells into monocyte-like variant cells, GKT3-1.3V. A remarkable increase in Mo1-positive cells was observed by adding rGM-CSF to the cell culture, thus suggesting the enhancement by rGM-CSF of the monocyte-like character of the variant cell.

Growth of Cells in Clonogenic Assay and Suspension Culture and Effects of r-GM-CSF, rM-CSF, and rG-CSF. The relation between the number of cells plated and the colonies yielded for both GKT3-1.3 and GKT3-1.3V is shown in Fig. 1. A statistically significant linear relationship was seen in GKT3-1.3, whereas a remarkable acceleration in clonogenicity with high cell density was observed in GKT3-1.3V. Considering the monocyte-like characteristics of GKT3-1.3V cells, we examined whether their growth was affected by the CSFs.

Fig. 2 discloses the short term growth curves of the GKT3-1.3V cells with or without 1 ng/ml of GM-CSF in suspension culture. The number of clonogenic cells increased exponentially for 7 days and then decreased thereafter. The addition of rGM-CSF partially inhibited the growth. In Fig. 3, a long term growth of cumulative clonogenic cells for 55 days, subcultured every week, with or without GM-CSF, is shown. As compared with controls, rGM-CSF (1 ng/ml) significantly suppressed the growth of the cells (P < 0.01), whereas M- (10 units/ml) and

Table 1 Percentage of positive cells of GKT3-1.3 and GKT3-1.3V cells for various surface marker antigens

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>GKT3-1.3</th>
<th>GKT3-1.3V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Growth</td>
<td>Growth</td>
</tr>
<tr>
<td>OKM5 (CD36)</td>
<td>2.7</td>
<td>93.9</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>14.8</td>
<td>98.3</td>
</tr>
<tr>
<td>Mo1 (CD11)</td>
<td>9.5</td>
<td>61.6</td>
</tr>
<tr>
<td>My7 (CD13)</td>
<td>4.3</td>
<td>41.5</td>
</tr>
<tr>
<td>My4 (CD14)</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Leu-7</td>
<td>52.2</td>
<td>2.1</td>
</tr>
</tbody>
</table>

G-CSF (10 ng/ml) did not inhibit the growth (data not shown). The dose-dependent curves for the effects of these CSFs on the survival of GKT3-1.3V cells both in methylcellulose and suspension are shown in Fig. 4. rGM-CSF significantly inhibited the growth of the cells in both culture systems in a dose-dependent manner, while rM-CSF and rG-CSF did not show any inhibitory effects. In contrast, none of the three CSFs affected the growth of the parent cell line GKT3-1.3 cells in any of the culture systems (data not shown).

Effect of rGM-CSF on the Cell Cycle of GKT3-1.3V Cells. According to the histograms of the cells in cell cycle phases (Fig. 5), the addition of rGM-CSF (1 ng/ml) for 3 days resulted in 17% reduction of the percentages of cells cycling in G2, M, and S. This suggested that the antiproliferative effect of rGM-CSF was due to a block in cell cycle progression in G0 or G1.

Evaluation of a Soluble Growth Inhibitor Secretion by GKT3-1.3V Cells. The above findings suggest that GM-CSF inhibits the growth of GKT3-1.3V cells directly. However, the possibil-
cells. Furthermore, these inhibitory effects of the conditioned media and incubated for 1 h at 25°C before plating. Anti-GM-CSF antibody (1:500 dilution) was added to the conditioned media at the concentration of 10% (v/v) in the clonogenic assay. In some experiments, these conditioned media were added at the concentration of 10% (v/v) in the clonogenic assay. In some experiments, anti-GM-CSF antibody (1:500 dilution) was added to the conditioned media and incubated for 1 h at 25°C before plating. The results are shown in Table 2. Media conditioned by GKT3-1.3V, without rGM-CSF, did not significantly inhibit colony growth as compared with the control media without either cells or rGM-CSF. In contrast, media conditioned with cells plus rGM-CSF exhibited inhibitory effects, but the inhibition was not significantly different from the conditioned media without cells. Furthermore, these inhibitory effects of the conditioned media were completely abolished by treatment with anti-GM-CSF antibody. As one of the possible soluble intermediary substance, TNF was measured in the medium conditioned at 96 h but proved negative (less than 0.2 unit/ml) by enzyme-linked immunosorbent assay (Asahi Chemical Industry, Shizuoka, Japan). These results deny the involvement of a soluble inhibitor in the growth inhibition but rather suggest a direct inhibitory effect on the rGM-CSF on the cells.

Receptors for GM-CSF. The direct function of rGM-CSF on the cells was strongly suggested by specific binding assay. The results of binding experiments are shown in Fig. 6 (inset) in which increasing concentrations of [125I]-GM-CSF were incubated with GKT3-1.3V cells. A plot of specific binding as a function of radioligand concentration indicates that the binding is dose dependent and saturable. Scatchard analysis shown in Fig. 6 (inset) is consistent with the presence of high affinity and low affinity binding sites. The data are in agreement with the presence of both high affinity and low affinity binding sites. The Kd values for the high affinity and low affinity binding sites were calculated by the dilution at each subculture. SD for each measurement was too small to be shown.
The present study was carried out to determine the biological characters of SCLC. For this purpose, the GKT3-1.3 cell line established from a metastatic lesion was utilized. After multiple passages, a subclone, GKT3-1.3V, was obtained. When we compared the biological characters of GKT3-1.3 and GKT3-1.3V cells, the variant cells had gained monocyte-like properties and a higher proliferative ability than the original cell line. The monocyte-like properties were demonstrated by changes in morphology, growth pattern, nonspecific esterase stain, and modulations in the expression of surface markers.

The previous report on the effects of γ-interferon and GM-CSF on the SCLC cell line (13) prompted us to study whether our variant cell line was affected by such CSFs. CSFs are important growth factors for the proliferation and differentiation of myeloid progenitor cells (24–26). On the other hand, anti-proliferative and terminal differential effects on myeloid cells (293–1000 per cell) or cell lines (20–450 per cell) (23). Increase in the surface marker Mo1-positive cells were shown by the addition of CSFs, particularly rGM-CSF. This change suggested the possibility that the cells have differentiated in a manner similar to that of monocyte development (27, 29).

The above findings in phenotypical analysis and the responsiveness to GM-CSF indicate the monocyte-like characteristics of GKT3-1.3V but may be insufficient to conclude that the cells are of myeloid origin, as proposed by Ruff et al. (13), since hematopoietic markers and also GM-CSF receptors have been found on a wide variety of human tumor cells, including SCLC cells (32), and GM-CSF is reported to affect the growth of other nonhemopoietic cells (33). The possibility still remains that monocytes cells contaminated in SCLC cells became dominant during multiple passages to express the monocyct character of the cell line. However, the variant cell line exhibited abnormally high proliferative ability and lacked adherent properties. Also in our previous study, we reported that in suspension culture for mononuclear cells from AML patients, contaminated monocytic cells from normal clone did not grow, while leukemic cells showed exponential growth (19). Therefore, it is more likely that the cell line has derived from the original clone.

GM-CSF has been described to support the growth of granulocyte-macrophage in vitro (34). On the other hand, GM-CSF has also been reported to inhibit the growth of monocyct line U937. In this case, the antiproliferative effect of GM-CSF was mediated by TNF secreted by the U937 cells (35, 36). In the present study, rGM-CSF inhibited dose dependently the growth of GKT3-1.3V cells in colony assay in methylcellulose as well as in suspension culture. We tried to determine the mechanism by which the growth of GKT3-1.3V cells was inhibited by GM-CSF. We tested whether the inhibition of the cell proliferation was mediated by some soluble factors secreted by the cells in the presence of GM-CSF. The antiproliferative effect of the conditioned media at various time points was completely reversed by the treatment with anti-GM-CSF antibody, excluding the involvement of a soluble intermediary substance, such as TNF. This was supported by the fact that TNF was not detected by enzyme-linked immunosorbent assay in the conditioned medium of a late phase. Our results may be explained by the direct activity of rGM-CSF on the cells, rather than an indirect activity mediated by other factors.

Subsequently, we studied the effect of GM-CSF on the cell cycle of GKT3-1.3V cell. GM-CSF brought about a decrease of cells in the S and G2-M phases (Fig. 5). This result suggests a block in G0 or G1, thus decreasing the rate of self-renewal.

The results presented above suggest that our variant cells may represent the clone within the tumor population, growing dominant through passages in vitro, and exhibiting monocyte-like characteristics. Their differentiation seems to be at the immature level where the growth is affected by GM-CSF but not by G- or M-CSF. Contrary to our result and the former study showing the antiproliferative effect of GM-CSF on SCLC lines (13), Munker et al. (37) reported that their SCLC lines were not affected by GM-CSF. These contradictory results indicate that the effect of GM-CSF may be different among various SCLC lines. The heterogeneity in differentiation level among SCLC cell lines may be one explanation for the differences in the reactivity to GM-CSF. Further studies are necessary to elucidate the mechanism producing different reactivities towards GM-CSF and to see whether or not the reactivities are dependent on the types of cell lines such as classic or variant types. These studies will lead to new modalities in treatment of the disease, such as utilizing differentiation-inducing CSFs in combination with chemotherapeutic agents.
EFFECT OF GM-CSF ON LUNG CANCER CELL LINE


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